Induction of progressive glomerulonephritis by podocyte-specific overexpression of platelet-derived growth factor-D

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Platelet-derived growth factor-D (PDGF-D), normally expressed in podocytes, mediates mesangial cell proliferation *in vivo*. To study this further, we created transgenic mice with podocyte-specific overexpression of PDGF-D. Hemizygous mice were grossly indistinguishable from wild-type littermates through 11 months of age; however, hemizygous mice older than 4 weeks commonly exhibited increased cell proliferation within the glomerular tuft. Many hemizygous mice also developed widespread segmental glomerulosclerosis and focal extracapillary proliferation with fibrin/fibrinogen deposition, extensive tubulointerstitial damage, proteinuria, and renal insufficiency. Electron microscopy found focal foot process effacement. Renal mRNA expression of podocin and nephrin, as well as the number of glomerular WT-1-positive cells, were significantly reduced in hemizygous compared to wild-type mice, indicating loss and/or dedifferentation of podocytes. PDGF-A, -B, and both PDGF receptor chain mRNAs, fibronectin, type IV collagen, RANTES, MCP-1, and CCR-2 mRNAs were all increased in the renal cortex of PDGF-D transgenic mice. Only 8.5% of newborn mice were homozygous overexpressors exhibiting a mortality rate of 37% at 4 weeks. Thus, podocyte-specific overexpression of PDGF-D caused mesangioproliferative disease, glomerulosclerosis, and crescentic glomerulonephritis. Hence, podocyte-specific growth factor overexpression can induce paracrine mesangial cell proliferation upstream of the filtration flow.

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The platelet-derived growth factor (PDGF) system consists of four PDGF chains: PDGF-A to -D. PDGF-A and -B are secreted as homo- or heterodimers and bind to and induce PDGF receptor dimers composed of α- and/or β-chains. Two novel PDGF isoforms, PDGF-C and -D, are released as homodimers only.[1–3] Whereas PDGF-BB is a ligand for all receptor types, PDGF-D binds to the PDGF ββ-receptor but not to the αα-receptor.[1,2] PDGF-D is produced as a latent factor. Proteolytic cleavage of a 'Clr/Cls', urchin endothelial growth factor-like protein, and bone morphogenic protein 1 (CUB) domain from each chain is required for its activation.[4] In addition, PDGF-D differs from PDGF-BB in that its C terminus lacks a basic amino-acid sequence that mediates binding to the extracellular matrix.[4] Recent studies have shown that PDGF-D induces mesangial cell proliferation *in vitro* and is overexpressed in mesangioproliferative glomerulonephritis *in vivo*.[5,6] Hepatic transfection with an adenoviral vector expressing PDGF-D led to elevated circulating levels of PDGF-D and induced prominent mesangioproliferative nephritis in mice, whereas antagonism of PDGF-D in a rat model of mesangioproliferative disease ameliorated the renal changes.[6,7] These four observations establish PDGF-D, similar to PDGF-B, as an important mediator of mesangioproliferative nephritis *in vivo*.

In the adult human kidney, PDGF-D is expressed by podocytes, arterial smooth muscle cells, some neointimal smooth muscle cells of arteriosclerotic vessels, and by smooth muscle cells of *vasa rectae* in the medulla.[8] In contrast, PDGF-D is not expressed in podocytes in normal (healthy) mouse kidney. The glomerular expression of the PDGF receptor β-chain is restricted to the mesangium and, to a lesser extent, parietal epithelial cells. In human podocytes, expression of the PDGF β-receptor chain is absent.[9,10] To gain more insight into the role of podocyte-derived PDGF-D, we generated a transgenic mouse with podocyte-specific overexpression of PDGF-D.

RESULTS

Generation of transgenic mice with podocyte-specific overexpression of PDGF-D

A targeting vector was constructed in which the full-length PDGF-D cDNA, tagged with enhanced green fluorescent

protein (EGFP), was driven by a 2.5-kb long fragment of the podocin promoter (Figure 1a). As the podocin promoter is only active in differentiated podocytes, which cannot be cultured and transfected successfully, we additionally created an expression plasmid in which PDGF-D/EGFP generation was driven by a β-actin promoter. Increased proliferation of embryonic mouse fibroblasts (NIH3T3) as a marker for biological activity of PDGF-D fusion protein was specifically induced only by conditioned media from HEK 293 cells that overexpressed PDGF-D/EGFP in comparison with control transfected cells (Figure 1b). Next, the targeting vector was injected into fertilized oocytes and the transgenic litters were identified by a PCR reaction (Figure 1c).

Hemizygous mice developed normally during 11 months and were macroscopically indistinguishable from wild-type littermates. The distribution between genders was normal but only 8% of the offspring from matings between hemizygous mice were homozygous (Table 1). Homozygous mice exhibited a mortality rate of 37% at 4 weeks.

Intrarenal transgenic PDGF-D/EGFP mRNA was detected by reverse transcriptase-PCR (RT-PCR) in isolated glomeruli (Figure 1d). There was also increased glomerular PDGF-D mRNA expression in transgenic mice in comparison with wild-type mice (Figure 1d). Western blotting analysis confirmed the expression of the transgenic PDGF-D/EGFP fusion protein by detecting the EGFP tag in glomeruli isolated from hemizygous nonproteinuric transgenic mice, but not in glomeruli of wild-type mice (Figure 1e). The protein detected displayed the same molecular weight as the PDGF-D/EGFP fusion protein when overexpressed in COS7 cells (positive control, Figure 1e), indicating that our transgenic mice express the full-length fusion protein enclosing PDGF-D and the EGFP tag. The size of the protein corresponded to the EGFP-tagged inactive PDGF-D monomer with a CUB domain. In contrast to nonproteinuric mice, in renal cortex of 8-week-old hemizygous proteinuric mice, we detected the active form of the transgenic PDGF-D/EGFP protein with a molecular weight of approximately 44 kDa (that is, EGFP-tagged PDGF-D without a CUB domain; Figure 1f).

By immunohistochemistry, the constitutive glomerular PDGF-D protein expression was easily detectable within mesangial cells (Figure 1g). At the age of 4 weeks, damaged glomeruli of transgenic mice contained occasional PDGF-D-positive cells in podocyte locations ($<5\%$ of the glomerular cells; Figure 1g). No PDGF-D was detectable in urine and serum samples by enzyme-linked immunosorbent assay (data not shown). In immortalized mouse podocytes, we demonstrated that both undifferentiated and differentiated podocytes were, in principle, able to express endogenous PDGF-D mRNA (Figure 1h).

Apart from renal cortex, transgene PDGF-D/EGFP mRNA expression was detected in the brain and bladder of hemizygous transgenic mice (Table 2) but not in the heart, lung, liver, muscle, colon, thymus, testis, or ovary. In a minority of animals, we observed transgene expression in the spleen, stomach, and uterus. Transgene expression in male and female mice was indistinguishable (Table 2). Although the transcriptional activity of the 2.5 kb fragment of the podocin promoter was described to be podocyte specific,[11] podocin mRNA expression was also detected in the brain but not in the bladder of wild-type mice ($n=7$, data not shown).

Transgenic mice develop proteinuria, glomerulosclerosis, and crescentic glomerulonephritis

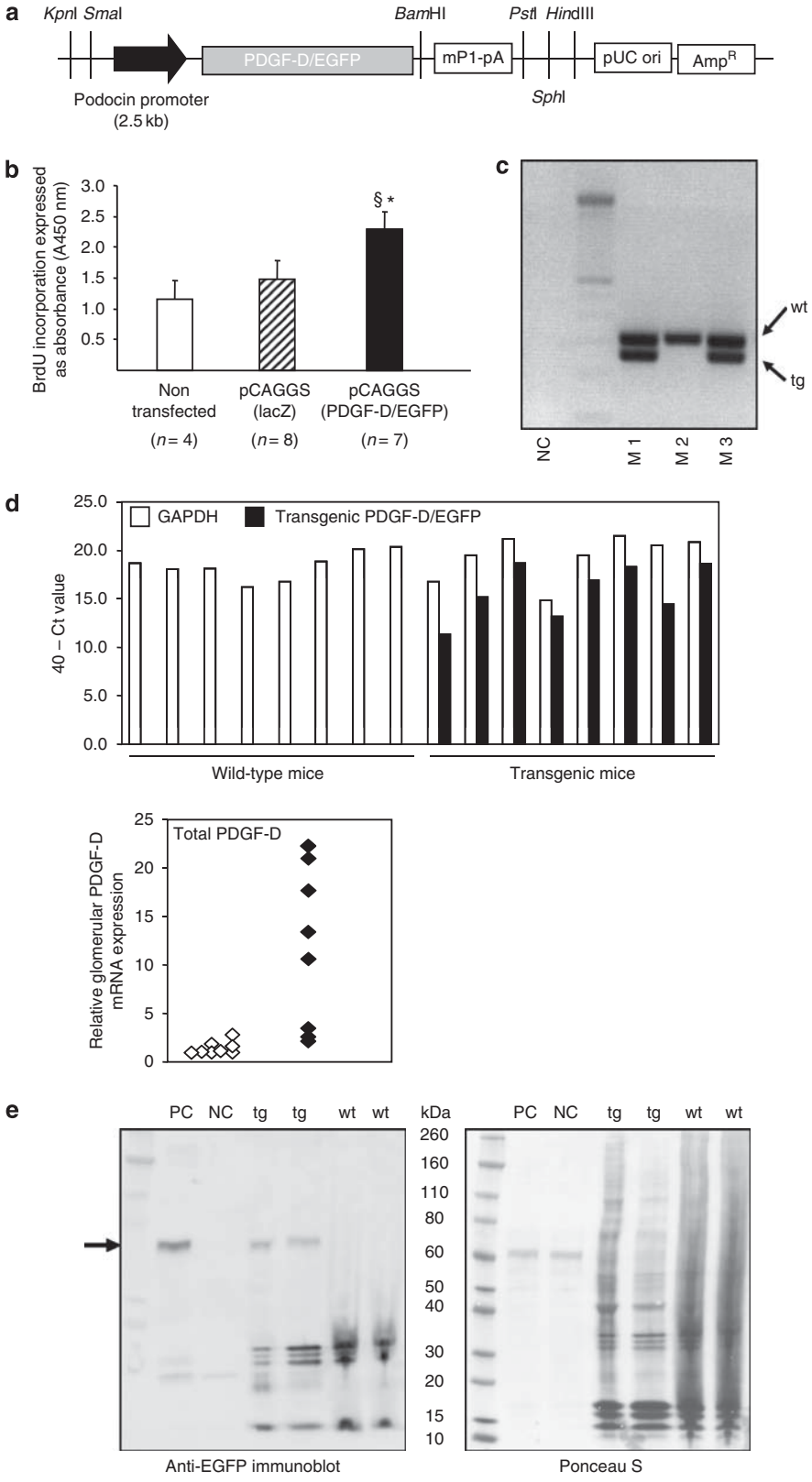
At the age of 7 weeks, 10 of 53 hemizygous mice, but no wild-type mouse, had developed proteinuria (Figure 2a). Up to 11 weeks of age, wild-type and hemizygous mice without proteinuria showed normal renal morphology; in particular, there was no evidence of increased mesangial cell proliferation or glomerulosclerosis. In contrast, proteinuric mice of three different founder lines aged 6–26 weeks (expression of the transgenic PDGF-D/EGFP of founder line F52 and F118 is shown in Figure 2c) exhibited severe glomerular injury, characterized by focal and segmental glomerulosclerosis and by focal extracapillary proliferation, as well as by tubulointerstitial damage (Figure 2b). Electron microscopy revealed widespread podocyte foot process effacement, as well as focal capillary collapse. Furthermore, a multilayered parietal epithelium indicative of focal extracapillary proliferation could be confirmed by electron microscopy (Figure 2d).

The development of proteinuria correlated with transgenic PDGF-D/EGFP protein expression in cortical tissue (Figure 3a). With respect to transgene mRNA, all hemizygous mice exhibited an increased PDGF-D mRNA expression in renal cortex tissue (Figure 3b); however, a significant difference in proteinuric vs nonproteinuric animals could only be observed in 4-week-old mice (Figure 3c).

The rare surviving homozygous mice expressed transgenic PDGF-D/EGFP and overexpressed PDGF-D mRNA in the renal cortex; however, in comparison with proteinuric hemizygous mice, their levels of transgenic PDGF-D/EGFP protein were decreased (Figure 3d, and e) and no renal phenotype developed (data not shown). This suggests that only homozygous mice with a low level of transgene translation were viable. To further analyze the phenotype of homozygous mice, 32 embryos from hemizygous breeding pairs were killed at day 19.5 of embryonic development. The genotype distribution followed the Mendelian ratio (16% wild type, 53% hemizygous, and 31% homozygous) and no obvious alterations in renal development or morphology could be observed up to this stage (Figure 3f), suggesting peripartal lethality from nonrenal causes.

To assess early stages of glomerular damage in this murine model, we analyzed kidneys from randomly selected 2-, 4-, 6-, and 8-week-old hemizygous PDGF-D transgenic mice. Whereas cortical renal transgene mRNA expression decreased in nonproteinuric mice, it increased in those with proteinuria (Table 3). Transgenic PDGF-D/EGFP protein levels were increased in proteinuric in comparison with nonproteinuric 4-, 6- and 8-week-old hemizygous mice (Figure 4a). Crescents occurred in proteinuric transgenic hemizygous

mice from the age of 4 weeks onward (Table 3). At the age of 2 weeks, glomeruli showed a normal morphology. Starting at 4 weeks, we observed an increasing mesangial cellularity when compared with wild-type littermates (Figure 4b). This was accompanied by a significant increase in proliferating, that is, proliferating cell nuclear antigen (PCNA)-positive, glomerular cells in proteinuric hemizygous mice in comparison with nonproteinuric hemizygous mice (Figure 4c).



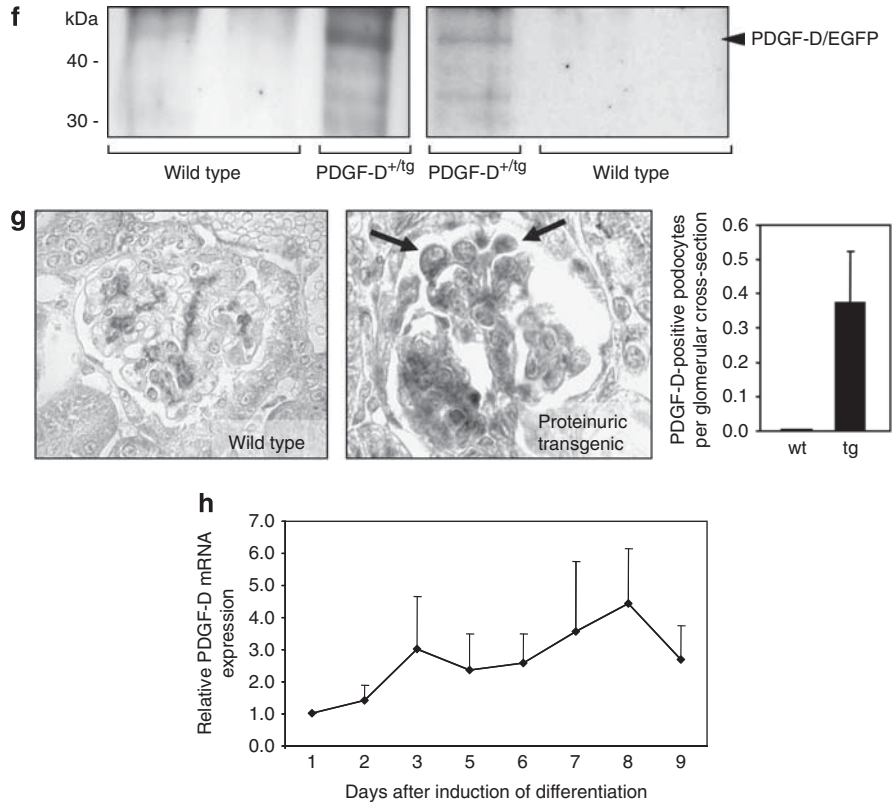


Figure 1 | Continued.

The number of 5-bromodeoxyuridine (BrdU)-positive cells localized inside the glomerular tuft was significantly increased in hemizygous proteinuric mice and mildly increased in nonproteinuric hemizygous mice, whereas the number of BrdU-positive podocytes, as well as the number of infiltrating T-lymphocytes, neutrophils, and macrophages, was unchanged

Figure 1 | Generation of a transgenic mouse strain with podocyte-specific overexpression of platelet-derived growth factor-D (PDGF-D). (**a**) Targeting vector injected into fertilized B6D2/F1Crl oocytes. The full-length PDGF-D cDNA, tagged with enhanced green fluorescent protein (EGFP) at the C terminus, is driven by a 2.5-kb fragment of the podocin promoter (Amp^R, ampicillin resistance; pUC ori, plasmid replication origin; mP1-pA, sequence (nucleotides +95 to +625 relative to CAP site, providing an artificial intron) and polyadenylation signal of the murine protamine (P1) gene). (**b**) Verification of the biological activity of the PDGF-D/EGFP fusion protein *in vitro*. HEK 293 cells were transfected with pCAGGS-lacZ or pCAGGS-PDGF-D and the supernatant was collected. NIH 3T3 cells were incubated with the conditioned media, or medium from untransfected cells, and the proliferation was measured. Data are means ± s.d. BrdU, 5-bromodeoxyuridine. $^*P<0.001$ (analysis of variance (ANOVA) with BONFERRONI *post hoc* test) vs nontransfected cells. $^{\S}P<0.001$ (ANOVA with BONFERRONI *post hoc* test) vs pCAGGS (lacZ)-transfected cells. (**c**) The transgenic litters were identified by a PCR reaction in which wild-type and transgenic PDGF-Ds were amplified (NC, negative control; M1, M2, M3, tail biopsies from three different littermates). (**d**) Transgene expression was also qualitatively detected by real-time reverse transcriptase-PCR (RT-PCR) using a PDGF-D/EGFP-specific probe in RNA from isolated glomeruli (upper panel). A ct value of 40 cycles indicates that the fluorescence signal did not reach the threshold until cycle 40, and therefore no PCR product was amplified. Data of eight wild-type and eight transgenic mice are shown. Total glomerular PDGF-D mRNA (that is, endogenous plus transgene mRNA) was detected by real-time RT-PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (lower panel). Glomerular PDGF-D mRNA is overexpressed in transgenic relative to wild-type mice (ct value, cycle of the real-time RT-PCR, in which the fluorescence signal reached the threshold). (**e**) Overexpressed EGFP protein could be detected by western blot analysis (arrow) using glomerular lysates of hemizygous transgenic or nontransgenic animals. Lysates of COS-7 cells transiently transfected with PDGF-D/EGFP or PDGF-D were used as positive and negative controls, respectively. Loading of the membrane was controlled by staining with Ponceau S (NC, negative control; PC, positive control; tg, transgene; wt, wild type). One representative blot out of two blots performed is shown. (**f**) Transgenic PDGF-D/EGFP protein was detected in the renal cortex of 8-week-old proteinuric hemizygous transgenic mice by pulldown of the protein with an anti-EGFP antibody and detection in a western blot using an anti-PDGF-D antibody. Results of two different transgenic mice and four wild-type controls are shown. (**g**) PDGF-D protein was detected by immunohistochemistry in proteinuric hemizygous transgenic and wild-type mice. In wild-type mice, PDGF-D is exclusively expressed by mesangial cells, whereas in proteinuric transgenic mice PDGF-D could be detected in the mesangium and infrequently in podocytes. In proteinuric hemizygous mice, PDGF-D-positive podocytes, that is, cells at the edge of the glomerular tuft, were counted. Podocytes expressing PDGF-D could not be detected in wild-type mice. (**h**) Transcript expression of PDGF-D during the differentiation of the immortalized mouse podocyte cell line WT5. Podocytes were switched to nonpermissive conditions, RNA was isolated ($n=6$ each) at the time points indicated, and endogenous PDGF-D mRNA expression was measured by real-time RT-PCR and normalized to GAPDH as an internal standard. Data are shown relative to undifferentiated podocytes (day 1).

(Figure 4d). An increased number of proliferating parietal cells could be observed in proteinuric but not in nonproteinuric hemizygous transgenic mice (Figure 4d).

Glomerular extracapillary proliferation (crescents) in the proteinuric PDGF-D transgenic hemizygous mice was characterized by fibrin/fibrinogen deposition (Figure 5a and b; Table 4) and the accumulation of type I collagen and fibronectin (Figure 5c and d and Table 4). The latter was accompanied by increased mRNA levels of fibronectin and type IV collagen in the renal cortex (Figure 5e). No *de novo* α-smooth muscle actin expression could be observed in the mesangium of glomeruli containing crescents (data not shown).

The phenotype described above was observed in 3 of 20 screened founder lines (Figure 2b). However, two lines became extinct, because of reduced fertility of one line and the restricted number of transgenic offsprings of the second founder line. In this second line, both transgenic offsprings developed proteinuria at the age of 7 weeks. Hemizygous, podocyte-specific PDGF-D transgenic mice of the third founder line (F90) were fertile, but homozygous mice were born infrequently (see above). All analyses were performed in littermates of the third founder line F90.

Podocytes are subject to damage in PDGF-D transgenic hemizygous mice

Electron microscopy in hemizygous proteinuric PDGF-D transgenic mice revealed widespread foot process effacement of the podocytes, whereas the morphology of glomerular endothelial cells and the basal membrane appeared normal (Figure 6a). In addition, the cortical mRNA expression of the podocyte-specific genes podocin and nephrin was reduced in comparison with wild-type mice (Figure 6b), as was the number of Wilms' tumor 1 (WT-1)-positive cells per glomerular cross-section (Figure 6c), suggesting that podocytes were damaged in hemizygous transgenic mice.

By TUNEL (TdT-mediated dUTP nick end labeling) staining, an increased number of apoptotic glomerular cells were detected in hemizygous proteinuric transgenic mice in comparison with wild-type mice. A fraction of glomerular apoptotic cells located at the glomerular edge indicating apoptotic podocytes (Figure 6d).

The renal phenotype seen in our transgenic mice was not due to an altered expression of members of the vascular endothelial growth factor (VEGF) system, as mRNA expression of total VEGF, VEGF receptors (KDR-1 or FLT-1), or the soluble inhibitory VEGF receptor-1 (sFLT-1; data not shown), as well as glomerular VEGF protein expression (Table 4), in hemizygous proteinuric transgenic mice was similar to that of wild-type controls.

Table 1 | Mendelian ratio of offspring from breeding between hemizygous mice

	Gender		Genotype		
Offspring	Male	Female	Wild type	Hemizygous	Homozygous
N=223	115 (52%)	108 (48%)	66 (30%)	138 (62%)	19 (8%)

Overexpression of PDGF-D in podocytes is associated with increased mRNA expression of PDGF isoforms/PDGF receptors

In transgenic hemizygous mice with proteinuria, the mRNA expression of PDGF-A and PDGF-B, as well as those of both PDGF receptor chains α and β, was increased (Figure 7). No changes of these mRNA species were observed in 4-, 6-, and 8-week-old healthy transgenic mice (data not shown). The mRNA expression of PDGF-C remained unchanged. As PDGF-D is a high-affinity ligand for the PDGF receptor β-chain, we stained for PDGF receptor β-chain protein in renal tissue. No obvious deviation from the normal mesangial and parietal epithelial cell receptor staining pattern was observed in diseased transgenic hemizygous mice (Figure 7b); however, the relative glomerular area staining positively for the β-chain was increased in proteinuric transgenic mice in comparison with wild-type mice (Figure 7b and Table 4).

Characterization of crescentic glomerulonephritis in transgenic mice

Crescentic glomerulonephritis is typically associated with a strong inflammatory process. Indeed, proteinuric transgenic hemizygous mice demonstrated increased renal cortical mRNA levels of proinflammatory chemokines MCP-1 (monocyte chemotactic protein-1) and RANTES (regulated upon activation, normal T-cell expressed, and secreted), as well as the chemokine receptor CCR-2 (chemokine (C-C motif) receptor 2), when compared with wild-type mice (Figure 8a). The increased mRNA expression of proinflammatory chemokines was associated with an increased accumulation of ER-HR3-positive macrophages within the multilayer parietal epithelium of injured glomeruli (Figure 8b and c).

Table 2 | Transgene mRNA expression in different tissues of hemizygous transgenic and wild-type mice

	Kidney	Heart	Lung	Liver	Muscle	Spleen	Brain	Colon	Stomach	Bladder	Bone marrow	Thymus	Testis	Ovary	Uterus
Transgenic															
Male	**9/9**	0/3	0/3	0/3	0/3	**2/9**	**10/10**	0/3	**1/12**	**12/12**	0/3	0/2	0/3		
Female	**10/10**	0/3	0/3	0/3	0/3	**4/9**	**8/8**	0/3	**4/11**	**11/11**	0/1	0/3		0/3	**4/11**
Wild type															
Male	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4		
Female	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3		0/3	0/3

Bold values indicate tissues in which the transgene was detected.

A high percentage of proteinuric mice showed an accumulation of IgA and IgM in glomeruli, which exhibited extracapillary proliferation (data not shown). In total, 30 and 40% of the proteinuric mice showed glomerular deposition of IgG and complement factor C3c, respectively (data not shown). With the exception of one animal, only mice with proteinuria exhibited immunoglobulin and C3c deposits.

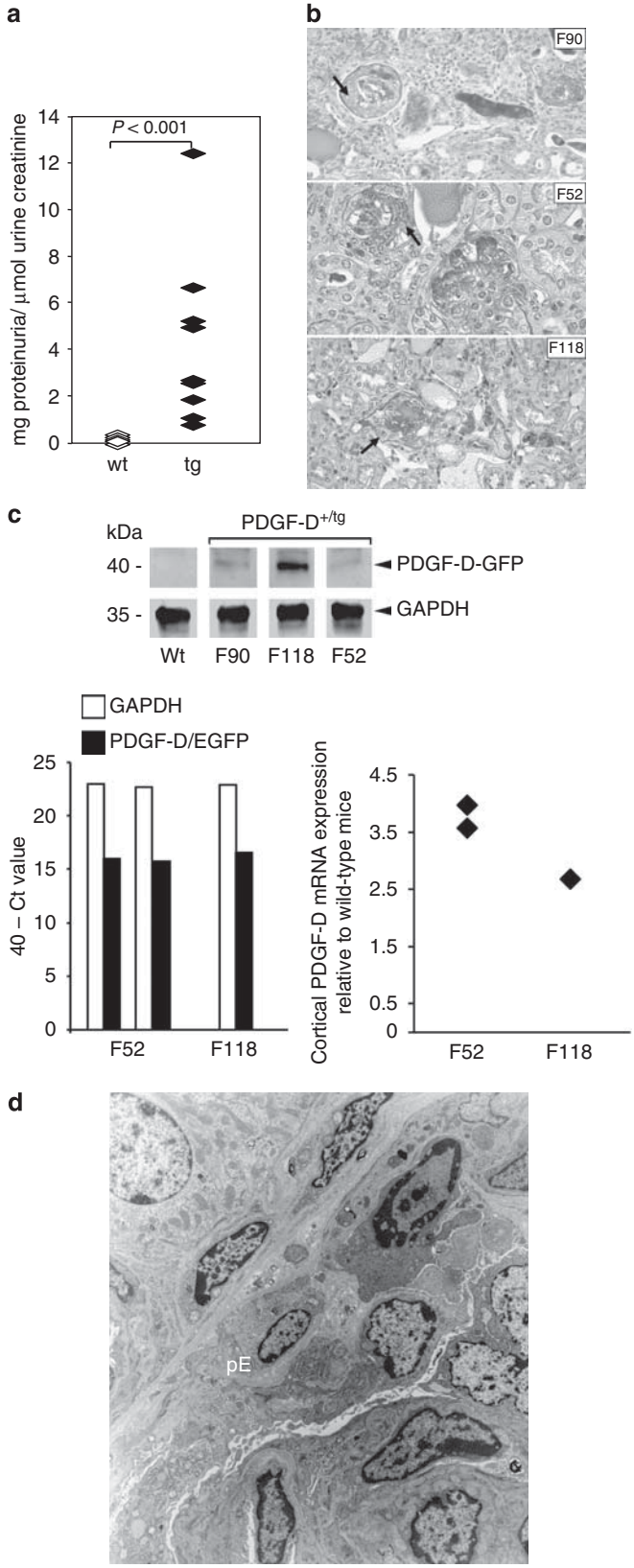


Figure 2 | Podocyte-specific overexpression of platelet-derived growth factor-D (PDGF-D) leads to increased proteinuria, glomerulosclerosis, and crescentic glomerulonephritis. (**a**) Proteinuria was measured in spontaneous urine samples ($n = 9$ proteinuric transgenic mice (five male mice (4–8 weeks old) and four female mice (4–7 weeks old)); $n = 9$ age- and gender-matched wild-type mice, $P < 0.001$ Mann–Whitney *U*-test). (**b**) Renal morphological changes in proteinuric transgenic mice. Glomerulosclerosis and crescentic glomerulonephritis were observed in periodic acid-Schiff (PAS)-stained renal sections. Representative findings in proteinuric transgenic mice were obtained from three different founder lines: F90 (8-week-old male mouse with glomerulosclerosis score 4), F52 (6-week-old male mouse, score 4), and F118 (26-week-old female mouse, score 2). Crescents are indicated by arrows. (**c**) Transgene expression was detected in founder lines F90 and F52 by anti-enhanced green fluorescent protein (anti-EGFP) western blot analysis and PCR analysis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (**d**) By electron microscopy, multilayer parietal epithelium could be observed in proteinuric transgenic mice. pE, parietal epithelia; tg, transgene; wt, wild type.

DISCUSSION

The major finding in our transgenic mouse model was the development of a proliferative glomerulonephritis. The podocyte-specific overexpression of PDGF-D resulted in proliferative changes within the glomerular tuft. Given that the number of podocytes, as well as infiltrating neutrophils, macrophages, and T lymphocytes, remained unchanged in the transgenic mice, the increased glomerular cell proliferation within the glomerular tuft appears to result from mesangial and/or endothelial cells, both of which can express PDGF β-receptors.[9,10] In addition, the number of proliferating parietal epithelial cells was significantly increased in proteinuric transgenic mice in comparison with nonproteinuric transgenic mice. Our data are in accordance with a mitogenic effect of PDGF-D in mesangial cells *in vitro*, as well as with the observation that systemic overexpression of PDGF-D using an adenoviral construct directing hepatic production of PDGF-D resulted in a mesangial proliferative glomerulopathy.[6,7] In addition, PDGF-B, a potent mesangial cell mitogen, and the PDGF β-receptor were overexpressed in the cortex of transgenic proteinuric mice. It is likely that this occurred in the course of mesangioproliferative changes within the glomerular tuft, as both mesangial and endothelial cells can express both PDGFs and PDGF receptors.[12] This process might have amplified the mitogenic activity of PDGF-D on mesangial cells, as well as on parietal epithelial cells, and it may thus have contributed to crescent formation as well.[12] So far, very little is known about a potential podocyte–mesangial cell crosstalk. Budde *et al.*[13] demonstrated that cell-culture medium conditioned by podocytes induced mesangial cell proliferation *in vitro*, but PDGF-B did not seem to be involved, as shown by competition

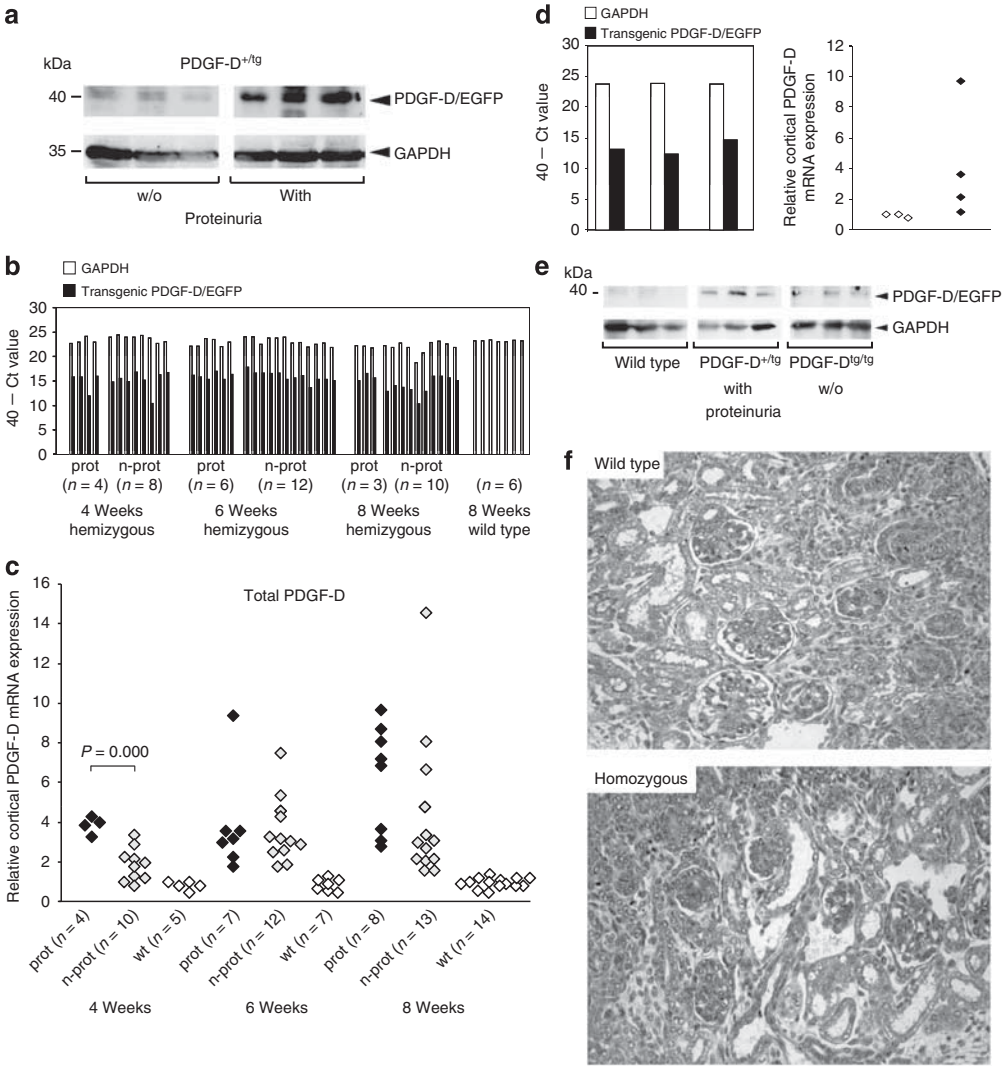


Figure 3 | Transgenic platelet-derived growth factor-D/enhanced green fluorescent protein (PDGF-D/EGFP) expression in proteinuric and nonproteinuric hemizygous and homozygous mice. (**a**) Increased levels of transgenic PDGF-D/EGFP protein were detected in proteinuric in comparison with nonproteinuric hemizygous transgenic mice (western blot analysis using an anti-EGFP antibody). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (**b**) Transgene expression was also qualitatively detected by real-time reverse transcriptase-PCR (RT-PCR) using a PDGF-D/EGFP-specific probe in RNA from cortical tissues of proteinuric and non-proteinuric hemizygous and wild-type mice (upper panel). A ct value of 40 cycles indicates that the fluorescence signal did not reach the threshold until cycle 40, and therefore no PCR product was amplified. (**c**) Total cortical PDGF-D mRNA (that is, endogenous plus transgene mRNA) was detected by real-time RT-PCR and normalized to GAPDH mRNA (lower panel). An increased cortical PDGF-D mRNA expression in proteinuric in comparison with nonproteinuric hemizygous mice could be observed in 4-week-old mice. In comparison with wild-type mice, hemizygous transgenic mice showed a cortical overexpression of PDGF-D mRNA (ct value, cycle of the real-time RT-PCR, in which the fluorescence signal reached the threshold; n-prot, nonproteinuric, prot, proteinuric, tg, transgene; wt, wild type). (**d**) Transgenic PDGF-D/EGFP could be detected in three homozygous mice using real-time RT-PCR. Cortical PDGF-D mRNA expression was increased in homozygous in comparison with wild-type mice. (**e**) Decreased transgenic PDGF-D/EGFP concentrations could be detected in surviving homozygous mice (3 weeks old) in comparison with proteinuric hemizygous mice by western blot analysis. (**f**) No changes in renal morphology could be observed in PAS-stained sections of homozygous transgenic mice in comparison with wild-type mice at the age of day 19.5 of embryonic development.

experiments. In addition, in that study, the maximal proliferation rate was reached after an incubation time of 48 h, whereas PDGF-DD and PDGF-BB induce maximal cell proliferation in mesangial cells after 24 h.[5] In contrast to the findings observed following hepatic overexpression of PDGF-D,[6,7] a considerable number of our transgenic hemizygous mice developed more severe glomerular injury, characterized by proteinuria, focal and segmental glomerulosclerosis, and by focal extracapillary proliferation, as well as by tubulo-interstitial damage and renal failure. The development of proliferative glomerulonephritis was not accompanied by an increased positive glomerular α-smooth muscle actin staining. This observation is comparable to the one in PDGF-BB-infused rats, where increased mesangial cell proliferation also occurred without *de novo* α-smooth muscle actin expression.[14]

In particular, the formation of extracapillary cellular proliferation with many features of crescents can be explained by paracrine effects of podocyte-derived PDGF-D. Thus,

Table 3 | Age-related alterations in proteinuric and nonproteinuric hemizygous transgenic mice

	Nonproteinuric					Proteinuric				
Age (weeks)	*n*	Proteinuria (mg/dl)	Transgenic PDGF-D/EGFP mRNA expression relative to 4-week-old mice	% Glomeruli containing crescents	Glomerulosclerosis score	*n*	Proteinuria (mg/dl)	Transgenic PDGF-D/EGFP mRNA expression relative to 4-week-old mice	% Glomeruli containing crescents	Glomerulosclerosis score
4	9	11 ± 6	1.0 ± 0.5	0	0	4	734 ± 574	1.0 ± 0.1	17 ± 10	1.8 ± 0.5
6	12	22 ± 21	1.1 ± 0.7	0	0	7	416 ± 272	1.6 ± 0.6	39 ± 24	3.0 ± 0.8
8	15	10 ± 6	0.4 ± 0.2	0	0	8	257 ± 174	1.9 ± 1.3	63 ± 17	3.5 ± 0.5

Abbreviations: EGFP, enhanced green fluorescent protein; PDGF-D, platelet-derived growth factor-D.

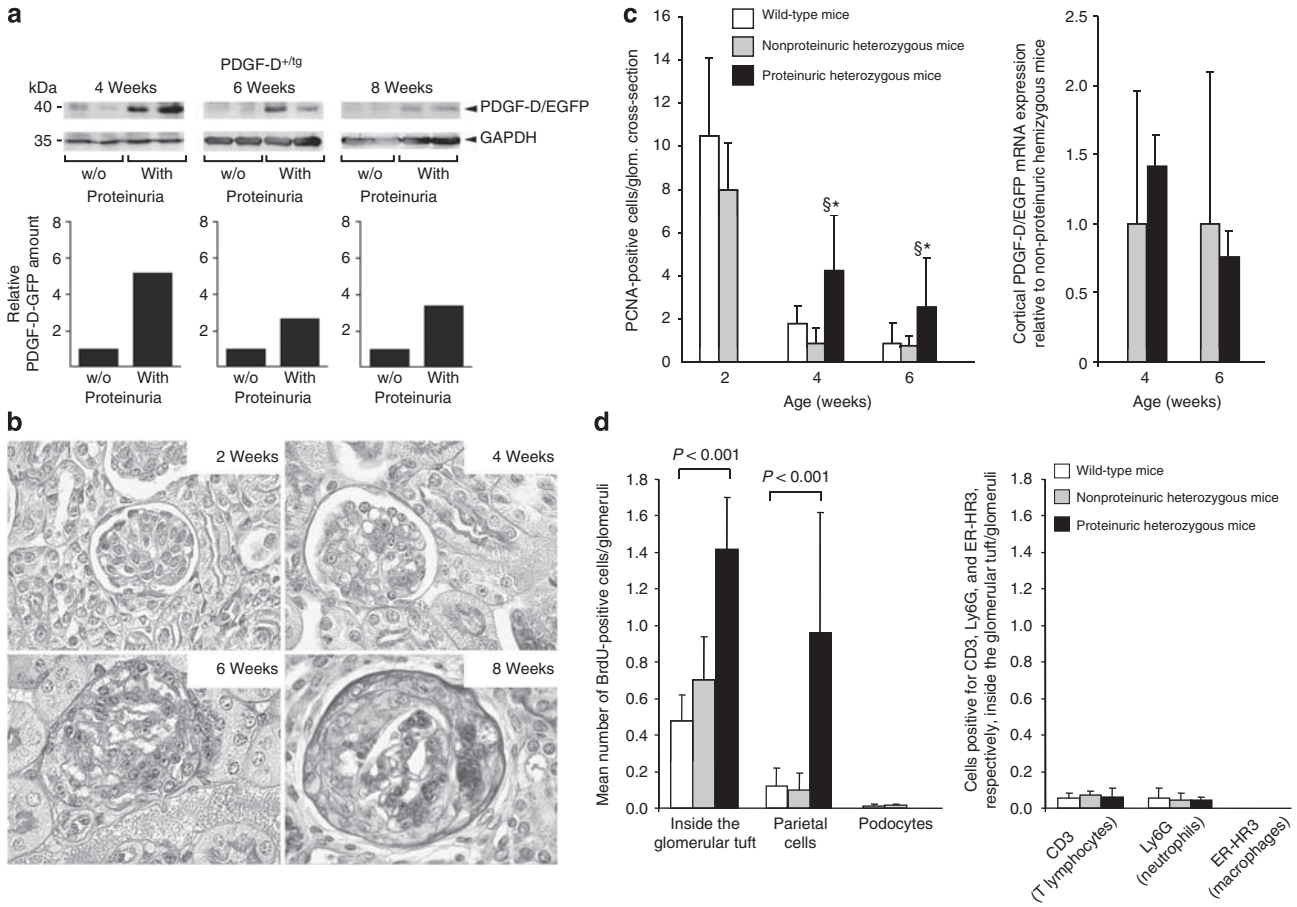


Figure 4 | Development of glomerulosclerosis and crescentic glomerulonephritis in transgenic mice. (**a**) Western blot analysis using an anti-enhanced green fluorescent protein (anti-EGFP) antibody from proteinuric and nonproteinuric hemizygous mice at the age of 4, 6, and 8 weeks. Increased transgenic platelet-derived growth factor-D (PDGF-D)/EGFP protein levels were observed in cortical tissues from proteinuric in comparison with nonproteinuric hemizygous mice. Mean values of both animals are shown in the morphometric analysis. (**b**) Renal periodic acid-Schiff (PAS)-stained sections from randomly selected transgenic mice at the age of 2, 4, 6, and 8 weeks. Increasing glomerular cellularity and the formation of extracapillary proliferation can be observed. (**c**) In proteinuric but not in nonproteinuric hemizygous mice, an increased number of glomerular proliferating cell nuclear antigen (PCNA)-positive cells can be observed. No significant alterations in PDGF-D/EGFP mRNA expression could be observed in proteinuric in comparison with nonproteinuric hemizygous mice (*$P < 0.05$ vs wild type; §$P < 0.05$ vs nonproteinuric hemizygous mice). (**d**) Randomly selected 6-week-old hemizygous transgenic ($n = 12$) and wild-type ($n = 6$) mice were labeled with 5-bromodeoxyuridine (BrdU) and the incorporated renal BrdU was detected by immunohistochemistry. An increased number of BrdU-positive cells could be observed inside the glomerular tuft in hemizygous nonproteinuric transgenic mice. Immunohistochemical analysis of CD3, Ly6G, and ER-HR3 showed no differences in the number of infiltrating immune cells inside the glomerular tuft in hemizygous nonproteinuric transgenic mice in comparison with wild-type mice.

parietal epithelial cells of Bowman's capsule have been described to express the PDGF β-receptor chain in humans and primates, as well as in cryoglobulinemic mice.[10,15] Similarly, the PDGF β-receptor chain has been detected in glomerular crescents.[16,17] Deposition of immunoglobulins and complement was exclusively observed in our proteinuric transgenic mice with extracapillary proliferation, but not in nonproteinuric mice, indicating that it may represent passive entrapment rather than the pathophysiological origin of the crescents.

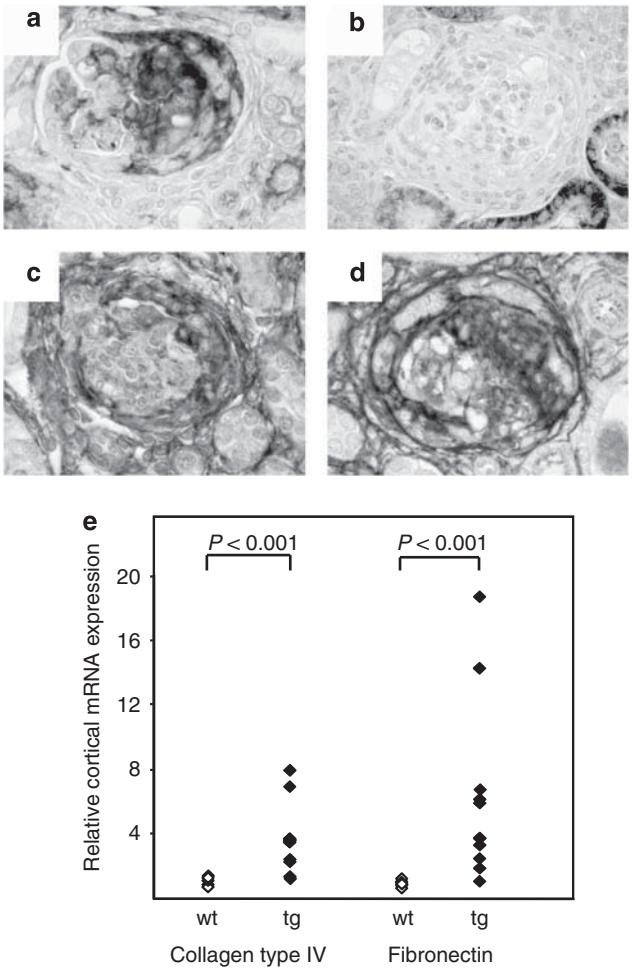


Figure 5 | Glomerular extracapillary proliferation in platelet-derived growth factor-D (PDGF-D) transgenic mice are characterized by deposition of fibrin/fibrinogen, type I collagen, and fibronectin. (**a**) Accumulation of fibrin/fibrinogen could be observed by immunohistochemistry in the extracapillary proliferation of the proteinuric transgenic mice. (**b**) Staining with a biotinylated goat anti-rabbit antibody confirmed the specificity of fibrinogen staining (negative control). By immunohistochemistry, (**c**) type I collagen and (**d**) fibronectin also showed an intense upregulation in the crescents (data are representative of $n = 10$ proteinuric transgenic and $n = 22$ age- and gender-matched wild-type mice). (**e**) Type IV collagen and fibronectin mRNA, as detected by real-time reverse transcriptase-PCR (RT-PCR) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The gene expression data from proteinuric transgenic ($n = 10$) and age- and gender-matched wild-type mice ($n = 6$) are shown (tg, transgene; wt, wild type); $P < 0.001$, Mann–Whitney *U*-test.

In our transgenic mice, podocyte-specific overexpression of PDGF-D resulted in a diminished mRNA expression of podocin and nephrin and a reduced number of glomerular WT-1-positive cells. These observations are in agreement with podocyte damage and correspond to findings, for example, in crescentic glomerulonephritis of other origin, where podocyte proliferation and the loss of differentiation markers, such as WT-1, synaptopodin, nephrin, and podocin, have also been observed.[18] The mechanism(s) by which transgenic expression of PDGF-D in podocytes results in

Table 4 | Immunohistochemical characterization of proteinuric hemizygous transgenic mice

Protein	Wild-type mice	Transgenic mice	*P*-value
PDGFR-β	3.3 ± 2.4	9.3 ± 4.1	0.016
Fibrin/fibrinogen	1.1 ± 1.1	16.2 ± 7.7	0.014
Fibronectin	3.3 ± 1.8	16.6 ± 7.7	0.009
Collagen type I	0.36 ± 0.2	9.7 ± 2.1	0.009
VEGF	0.95 ± 0.8	0.64 ± 0.3	Not significant

Abbreviations: PDGFR-β, platelet-derived growth factor receptor-β; VEGF, vascular endothelial growth factor.
Numbers depict percent positively stained glomerular area relative to the total glomerular area. Parietal epithelium was excluded in this analysis. *n*=5 proteinuric (4–8 weeks old) and *n*=5 wild-type mice (4–8 weeks old).

downregulation of slit diaphragm protein mRNAs currently remains unknown, but could involve podocyte apoptosis. The existence of apoptotic podocytes in renal disease accompanied by downregulation of nephrin expression and a decreased number of glomerular WT-1-positive cells is well established in other transgenic mouse models,[19] and indeed the inactivation of podocyte proteins such as podocin may be causal in this process.[20] Importantly, we were able to exclude the fact that the phenotype of the transgenic mice was secondary to disturbances of the VEGF system. Thus, Eremina *et al.*[21,22] demonstrated that podocyte VEGF is required for mesangial cell survival and differentiation, as well as for endothelial cell migration, differentiation, and survival.

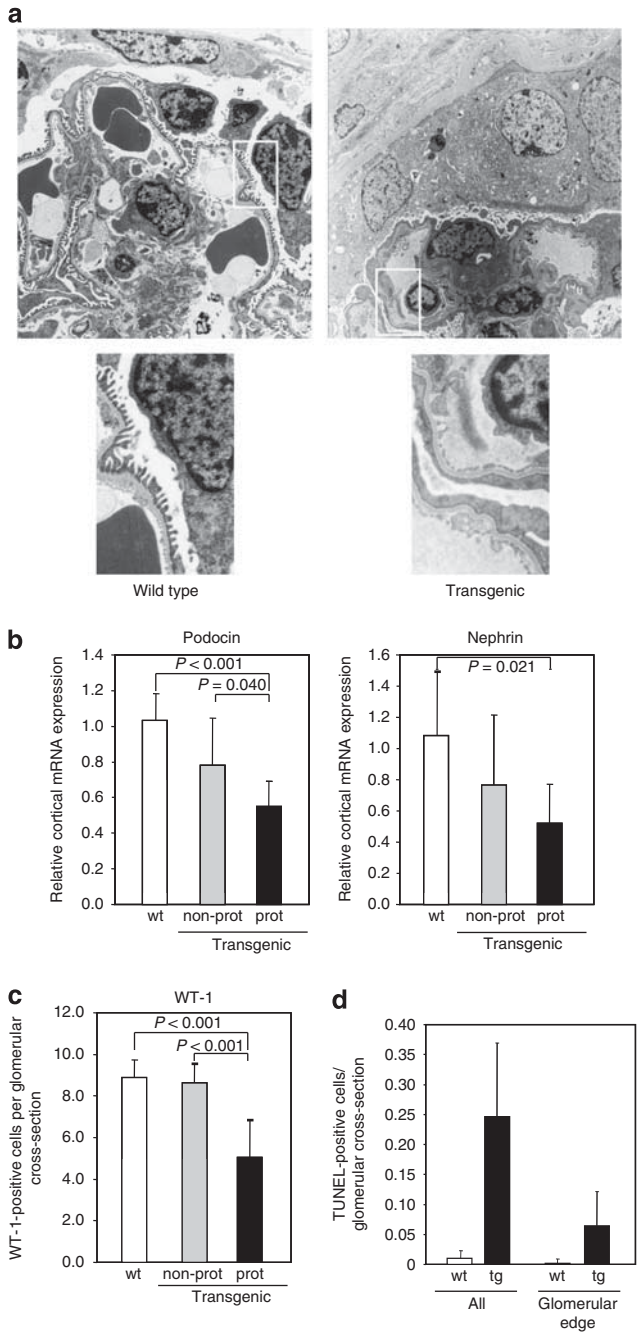
One notable feature of our transgenic model is that the renal phenotype occurred with expression of full-length PDGF-D in podocytes. Full-length PDGF-D requires proteolytic cleavage of its CUB domain to become biologically active.[1,2] Whereas plasmin exhibits such cleaving activity *in vitro*, the protease(s) involved in PDGF-D cleavage *in vivo* likely includes urokinase-type plasminogen activator, which was shown to be expressed in podocytes *in vitro*.[23,24]

In our mouse model, the transgene was driven by a fragment of the podocin promoter, which has been reported to be podocyte specific.[11] Genetic mosaicism may have contributed to the variable phenotype that we observed in heterozygote mice. The fact that most homozygous transgenic mice apparently died *in utero*, whereas other homozygotes survived long term with no renal phenotype, also supports the assumption of genetic mosaicisms and/or variable transgene penetrance.

Besides glomerular transgene expression, we detected the PDGF-D/EGFP mRNA in the brain and bladder. Contrary to prior reports,[11] and similar to other 'podocyte-specific' genes such as nephrin and synaptopodin,[25,26] we detected podocin mRNA expression in the brain of wild-type mice, whereas the bladder was negative. This latter observation suggests that the detection of the transgene mRNA expression in the bladder might have resulted from detached transgenic podocytes. Indeed, loss of podocytes in our transgenic mice was indicated by the decreased number of glomerular WT-1-positive cells and the reduced mRNA expression of podocin and nephrin. It appears very unlikely that the expression of

the transgene in the brain accounted for the findings of the present study as we consistently failed to detect PDGF-D in sera of transgenic animals (data not shown).

The same renal phenotype was observed in three different founder lines. This suggests that the phenotype is related to podocyte-specific overexpression of PDGF-D and not by the destruction of any other gene. Final proof for the pathophysiological role of PDGF-D in our mice was sought after by performing a rescue experiment using injections of an inhibitory anti-PDGF-D antibody from 3 weeks of age. However, this experiment failed (data not shown) because of the high variability of the mouse phenotypes, which in turn likely relates to the genetic mosaic (see above).



In conclusion, we present a novel model of murine glomerulonephritis in which podocyte-specific overexpression of PDGF-D leads to mesangioproliferative disease, glomerulosclerosis, and crescentic glomerulonephritis. More generally, we provide the first evidence that podocyte-specific overexpression of a growth factor is capable of inducing paracrine cell proliferation inside the glomerular tuft upstream of the filtration flow, as well as proliferation of parietal glomerular epithelial cells. It is noteworthy that mesangial expansion can accompany podocyte damage in focal segmental glomerulosclerosis.[27] This supports the hypothesis that podocytes may locally alter their PDGF release in response to injury, and that this podocyte-derived PDGF can induce intraglomerular pathology.

MATERIALS AND METHODS

Generation of the transgenic mice strain

All studies were approved by the local institutional review board. Full-length rat PDGF-D cDNA was amplified in a RT-PCR reaction from RNA isolated from rat adrenal gland tissue using the following primers (forward: 5′-GGGGTACCCCATGCACCGGCTCATCTTAG TC-3′; reverse: 5′-TCCCCCGGGGGAATCGAGGTGGTCTTGAG CTG-3′). The PCR fragment was digested with the restriction enzymes *Kpn*I and *Sma*I (both from MBI Fermentas, Burlington, ON, Canada) and cloned into the *Kpn*I/*Sma*I-digested vector pEGFP-N1 (Invitrogen Groningen, The Netherlands). PDGF-D cDNA tagged with EGFP was amplified (forward primer: 5′-ATGCA CCGGCTCATCTTAGTC-3′; reverse primer: 5′-CATGCCATGGCAT GTTACTTGTACAGCTCGTCCAT-3′) and ligated into the *Nco*I (New England Biolabs, Frankfurt a.M., Germany)-digested vector pSNlacZ, which contained a 2.5-kb long fragment of the podocin promoter.[11] The PDGF-D sequence was checked for mutations by sequencing after each cloning step. The targeting vector was digested with *Kpn*I and *Sph*I (New England Biolabs); the DNA fragment containing the podocin promoter and the PDGF-D/EGFP sequence was purified and injected into fertilized B6D2/F1Crl oocytes. Transgenic litters were identified using a PCR reaction from DNA

Figure 6 | Podocyte-specific overexpression of platelet-derived growth factor-D (PDGF-D) results in damage of podocytes. (**a**) Electron microscopy showed widespread foot process fusion in proteinuric transgenic mice. The white frame indicates the region, which is shown at higher magnification. (**b**) Real-time reverse transcriptase-PCR (RT-PCR)-based expression data of podocin and nephrin normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The figure depicts the transcript expression in proteinuric transgenic ($n = 10$), nonproteinuric transgenic ($n = 11$), and wild-type mice ($n = 6$ age- and gender-matched mice); $P < 0.001$ analysis of variance (ANOVA), Tukey's *post hoc* test. (**c**) Immunohistochemical analysis of Wilms' tumor 1 (WT-1) showed a decreased number of glomerular WT-1-positive cells in proteinuric transgenic in comparison with age- and gender-matched wild-type and nonproteinuric hemizygous mice ($n = 10$ proteinuric transgenic, $n = 16$ nonproteinuric transgenic, and $n = 22$ wild-type mice; non-prot, nonproteinuric; prot, proteinuric; wt, wild type), $P < 0.001$ ANOVA, Tukey's *post hoc* test. (**d**) TdT-mediated dUTP nick end labeling (TUNEL) staining of hemizygous proteinuric transgenic (tg; $n = 10$) and wild-type (wt) mice ($n = 10$). Apoptotic cells were counted in the whole glomerular tuft ('all') and at the glomerular edge.

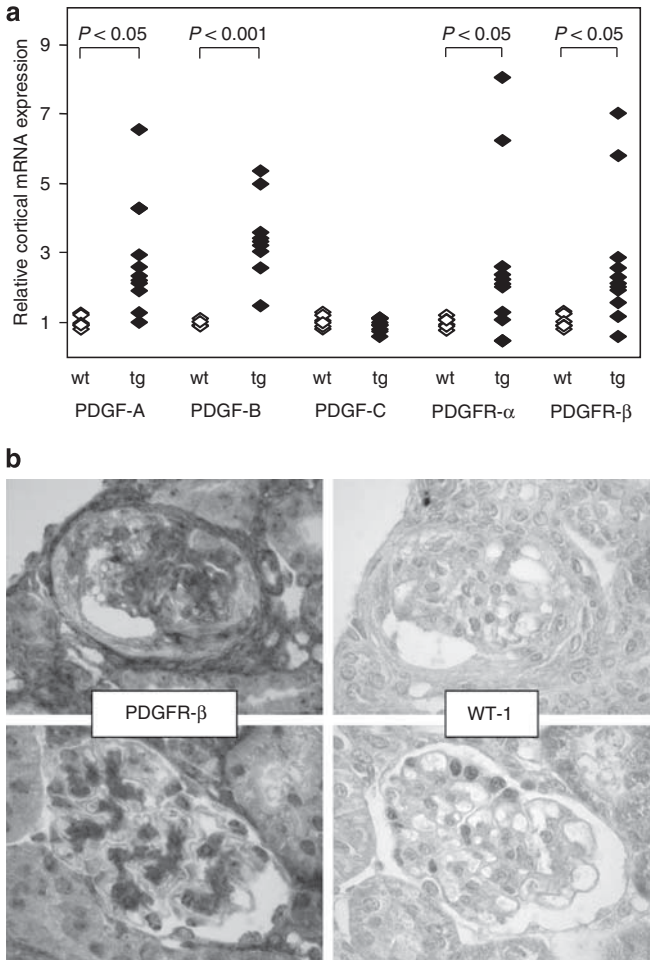


Figure 7 | Renal cortical mRNA expression of platelet-derived growth factor (PDGF) isoforms and PDGF receptor chains. (**a**) Podocyte-specific overexpression of PDGF-D induced overexpression of PDGF-A and -B, as well as both PDGF receptor chains, as measured by real-time reverse transcriptase-PCR (RT-PCR; data normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA). Gene expression data from proteinuric transgenic ($n = 10$) and age- and gender-matched wild-type mice ($n = 6$) are shown (tg, transgene; wt, wild type); $P < 0.001$ Mann–Whitney *U*-test. (**b**) PDGFR-β protein and the podocyte marker Wilms' tumor 1 (WT-1) were detected in serial sections from proteinuric hemizygous mice by immunohistochemistry. In sclerotic glomeruli, an increased PDGFR-β positively stained glomerular area accompanied by a reduction or absence of WT-1-positive glomerular cells could be observed. A normal PDGFR-β staining pattern and a normal number of WT-1-positive glomerular cells were detected in unaffected glomeruli of the same animal. A representative image of a proteinuric hemizygous mouse is shown.

isolated from tail biopsy using the following primers detecting the wild-type (forward: 5′-GGGGTACCCCATGACTCCCAGGAATCAC TCGGT-3′; reverse: 5′-TCCCCGCGGGGATCGAGGTGGTCTTGAG CTGCA-3′) and the transgenic PDGF-D (forward: 5′-CAGCGACTC CACAGGGACT-3′; reverse: 5′-GTGCCTGTCACCCGAATGTTC-3′). The transgenic mice were bred for two generations with C57BL/6 wild-type mice before analysis. Hemizygous and homozygous animals were distinguished by real-time PCR with EGFP primers and an EGFP probe (Table 5) by amplifying DNA isolated from tail biopsies.

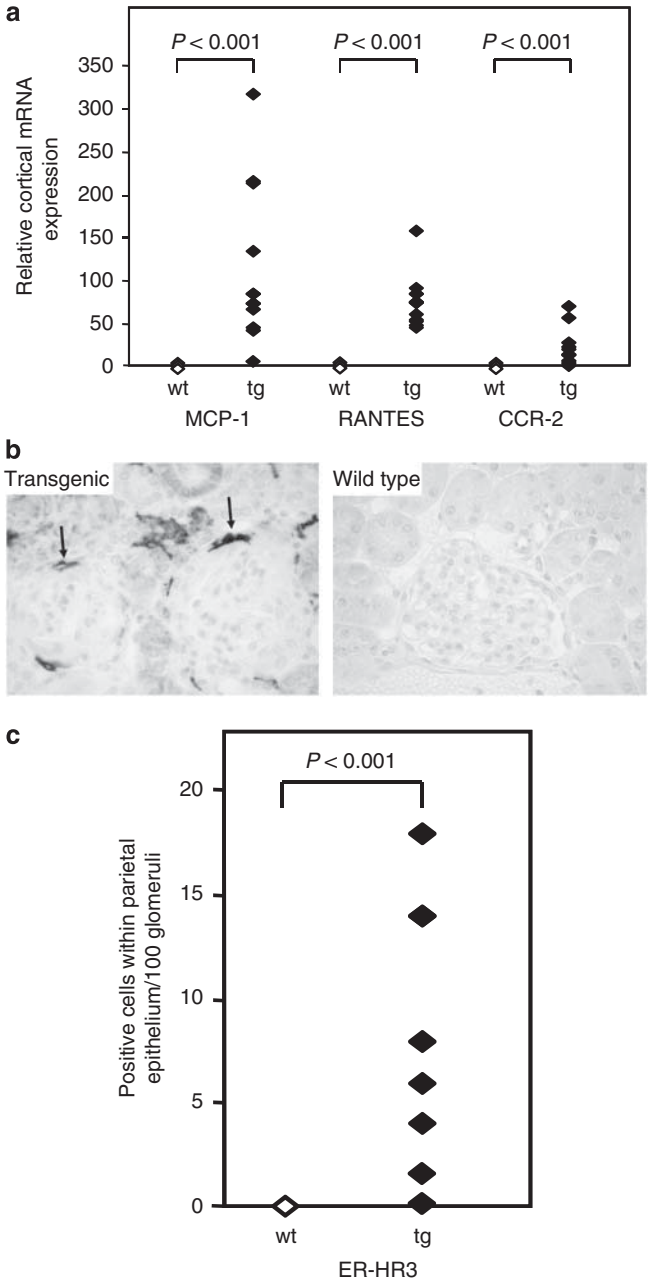


Figure 8 | Renal cortical mRNA expression of immune mediators and infiltration of immune cells. (**a**) Real-time reverse transcriptase-PCR (RT-PCR)-based expression data of MCP-1 (monocyte chemotactic protein-1), RANTES (regulated upon activation, normal T-cell expressed, and secreted), and CCR-2 (chemokine (C-C motif) receptor 2) normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The gene expression data from proteinuric transgenic ($n = 10$) and age- and gender-matched wild-type mice ($n = 6$) are shown; $P < 0.001$ Mann–Whitney *U*-test. (**b**) In crescents of proteinuric transgenic hemizygous mice, but not in parietal epithelium of wild-type mice, ER-HR3-positive cells (arrows) could be detected by immunohistochemistry. (**c**) In extracapillary proliferates, but not in parietal epithelium of wild-type mice, ER-HR3-positive cells could be observed. In this analysis, the glomerular tuft was not included (tg, transgene; wt, wild type; $n = 10$ proteinuric transgenic, and $n = 22$ age- and gender-matched wild-type mice); $P < 0.001$ Mann–Whitney *U*-test.

Table 5 | Primers for real-time RT-PCR

Primers for detection with SybrGreen (qPCR Core Kit for SYBR Green I)

Gene	Forward primer	Reverse primer
GAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC
Nephrin	ACCCTCCAGTTAACTTGTCTTTGG	ATGCAGCGGAGCCTTTGA
MCP-1	TGGCTCAGCCAGATGCAGT	ATTGGGATCATCTTGCTGGTG
RANTES	AGTGCTCCAATCTTGCAGTCG	CACTTCTTCTCTGGGTTGGCA
CCR-2	TGTGATTGACAAGCACTTAGACCA	ATGACAGGATTAATGCAGCAGTGT
VEGF (total)	TCTTCAAGCCGTCCTGTGTG	CTCCAGGGCTTCATCGTTACA
KDR-1	TGGATTCCTACCAGTATGGGACC	TCTAGCTGCCAGTACCACTGGA
FLT-1 (total)	CTGTCCATGAAAGTGAAGGCC	CATGTACCAAATAGCGAGCAGACT
sFLT-1	GGTGAGCACTGCGGCAAAAAG	TTAATGTTTGACATGACTTTGTGTGG

Primers for detection with Taqman probe (qPCR Core Kit)

Gene	Forward primer	Reverse primer	Probe
GAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC	AAGGCCGAGAATGGGAAGCTTGTCATC
PDGF-A	ACTTCCTGATCTGGCCCCC	TGAAGGCTGGCACTTGACG	TGTGGAGGTGAAGCGCTGCACTG
PDGF-B	CCATCCGCTCCTTTGATGAT	AAGTCCAGCTCAGCCCCAT	CGCCTGCTGCACAGAGACTCCGTA
PDGF-C	CTGGTGTGGAGATTAGTTGCAGTAG	CCAGCCCAAATCTCTCATCAA	TGAAAATGTGCGGATCCAGCTGACA
PDGF-D	GACACTTTTGCGACTCCGC	TGTGAGGTGATTGCTCTCATCTC	TTGCGCAATGCCAACCTCAGGA
PDGFR-á	AACGGAACCTTCAGCGTGG	AACTCGCTGGTCTTGAACGTC	CCTTACATCTGTGAGGCCACCGTCAAA
PDGFR-â	GAGGCTTATCCGATGCCTTCT	AAACTAACTCGCCAGCGCC	CCTGTGGCTCAAGGACAACCGTACCTT
PDGF-D/EGFP	CATGGTGGCGACCGGT	CAGTTGGATCATCATGAGCGA	AATCGAGGTGGTCTTGAGCTGCAGATG
Col4a1	GGCGGTACACAGTCAGACCAT	TGGTGTGCATCACGAAGGA	TCCGCAGTGCCCTAACGGTTGGT
Fibronectin	GATGGAATCCGGGAGCTTTT	TGCAAGGCAACCACACTGAC	CCGGCCTGAGGCCCTGCAG
Podocin	CAGCTGGGCTTCAGCACTCT	CCGCACTTTGGCCTGTCTT	TGGCGGTGGAAGCTGAGGCAC
EGFP	TACCTGAGCACCCAGTCCG	CTTGTACAGCTCGTCCATGCC	AAAGACCCCAACGAGAAGCGCGATC

Abbreviations: *CCR-2, chemokine (C-C motif) receptor 2*; *Col4a1, collagen, type IV, alpha 1*; *EGFP, enhanced green fluorescent protein*; *FLT-1, fms-related tyrosine kinase 1*; *GAPDH, glyceraldehyde 3-phosphate dehydrogenase*; *KDR-1, kinase insert domain receptor-1*; *MCP-1, monocyte chemotactic protein-1*; *PDGF, platelet-derived growth factor*; *PDGFR, platelet-derived growth factor receptor*; *RANTES, regulated upon activation, normal T-cell expressed, and secreted*; RT-PCR, reverse transcriptase-PCR; *sFLT-1, soluble fms-like tyrosine kinase-1*.
All sequences are listed in 5′ to 3′ direction.

Verification of the biological activity of the PDGF-D/EGFP fusion protein

HEK 293 cells were transfected with pCAGGS (PDGF-D/EGF), in which the PDGF-D/EGFP was driven by a β-actin promoter, or the control plasmid pCAGGS (lacZ) using Fugene Reagent (Roche, Basel, Switzerland). The supernatant was removed 40 h after transfection and treated with 0.5 U/ml plasmin for 20 min and 7.6 μg/ml aprotinin for 7 min. NIH3T3 cells were seeded in 96-well plates (Nunc, Wiesbaden, Germany), grown to subconfluence, and growth-arrested for 48 h in MCDB medium (Sigma-Aldrich, Taufkirchen, Germany). After 48 h, conditioned media were added and the cells were incubated for 27 h. DNA synthesis was determined during the last 21 h by a BrdU incorporation assay according to the manufacturer's instructions (Roche, Mannheim, Germany).

Differentiation of an immortalized mouse podocyte cell line

The immortalized mouse podocyte cell line WT5 was cultivated under permissive conditions at 33 °C as described.[28] Differentiation was induced by incubating the cells under nonpermissive conditions (37 °C; without γ-interferon).

Real-time quantitative RT-PCR

Glomeruli were isolated as described by Takemoto *et al.*[29] Total RNA was isolated from renal cortex or isolated glomeruli using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantification of the RNA content and sample purity, cDNA synthesis, and real-time quantitative RT-PCR were assessed and performed, respectively, as described.[6] All quantitative data from the real-time RT-PCR were normalized using GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as an internal control and calculated using the ΔΔct-method. The primer sequences are listed in Table 5. GAPDH cDNA amplification was used as an internal standard. In the analyses, 10 transgenic (5 male mice 4–8 weeks old and 5 female mice 4–7 weeks old) and 6 wild-type mice (2 male animals aged 4 and 8 weeks and 4 female animals aged between 4 and 6 weeks) were included.

Renal morphology and immunohistochemical analysis

Urinary protein concentrations were measured using the VITROS Chemistry UPRO Slides and the VITROS chemistry products calibrator kit 10 in the 'Vitros 250' system (OrthoClinical Diagnostics, Neckargemuend, Germany). In all, 10 hemizygous transgenic mice with urinary protein concentrations >100 mg/dl (5 male mice (4–8 weeks old) and 5 female mice (4–7 weeks old)) and 22 randomly selected wild-type mice (12 male animals aged 4–8 weeks and 10 female animals aged between 4 and 8 weeks) were used for the immunohistochemical analyses. Cohorts of 7 mice (4 hemizygous and 3 wild type) aged 2 weeks, 18 mice (4 proteinuric and 9 nonproteinuric hemizygous and 5 wild type) aged 4 weeks, and 29 mice (7 proteinuric and 12 nonproteinuric hemizygous and 10 wild type) mice aged 6 weeks were analyzed for glomerular PCNA-positive cells. An additional 18 mice (12 hemizygous and 6 wild type) aged 6 weeks were injected intraperitoneally with BrdU (100 mg/g body weight, Sigma) on three successive days before killing.

Tissue for light microscopy was fixed in methyl Carnoy's solution and embedded in paraffin. Sections (4 µm) were stained with periodic acid-Schiff reagent and counterstained with hematoxylin. Immunohistochemical analyses were performed following a previously published protocol.[30] Renal tissues were stained using the following antibodies:

- rabbit polyclonal anti-human fibrinogen (A0080, Dako, Hamburg, Germany),
- rabbit polyclonal anti-rat fibronectin (AB 1954, Chemicon, Hampshire, UK),
- goat polyclonal anti-human collagen type I (1310-01, Southern Biotech, Birmingham, AL),
- mouse monoclonal anti-human α-smooth muscle actin, horseradish peroxidase-tagged (clone 1A4, Dako),
- rabbit polyclonal anti-human WT-1 antibodies (C-19, sc-192, Santa Cruz, Santa Cruz, CA),
- rat polyclonal anti-human anti-CD3 (MCA1477, Serotec, Oxford, UK),
- rat monoclonal anti-mouse anti Ly-6G (RB6-8C5, BD Bioscience, San Jose, CA),
- rat monoclonal anti-mouse anti-ER-HR3 (T2012, Biomedicals, Augst, Switzerland),
- rabbit polyclonal anti-human IgG (F0202; Dako),
- rabbit polyclonal anti-human IgA (A0262; Dako),
- rabbit polyclonal anti-human IgM (F0203; Dako),
- rabbit polyclonal anti-human C3c (F0201; Dako),
- mouse monoclonal anti-human PCNA, biotinylated (PC-10, Oncogene, San Diego, CA), and
- mouse monoclonal anti-BrdU (BMC9318; Chemicon).

Negative controls for the immunohistochemical procedures consisted of substitution of the primary antibody with nonimmune IgG.

Glomerulosclerosis scores were determined in periodic acid-Schiff-stained sections as follows: score 0, no glomerulosclerosis; score 1, 0.1–25% of the glomerular area with sclerotic changes; score 2, 25.1–50%; score 3, 50.1–75%; and score 4, 75.1–100%. For WT-1, CD3, Ly-6G, ER-HR3, and PCNA staining, positively stained cells per 50 randomly selected glomerular cross-sections were counted. For the quantification of positively stained glomerular areas, renal tissues were evaluated by computer-based morphometry using the analysis v3.1 software (Soft imaging System GmbH, Münster, Germany). In all analyses, the investigator was unaware of the origin of the slides.

Electron microscopy

Tissue for electron microscopy was fixed in half-strength Karnovsky's solution (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer, pH 7.0). After fixation, the tissue was postfixed in 1% osmium tetroxide for 2 h, dehydrated in graded ethanols, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips 410 electron microscope (Phillips Export, Eindhoven, The Netherlands). Kidney tissue examined included at least two and usually three or more glomeruli per animal, as well as cortical and medullary interstitium, tubules, and blood vessels.

Immunoprecipitation and western blot analysis

Protein extracts from kidneys of transgenic or wild-type mice were obtained as described before.[31] Before immunoprecipitation, anti-GFP antibody (Alpha Diagnostic, San Antonio, TX) was covalently linked to Protein A Sepharose beads. For this, 20 µg of monoclonal antibody was incubated with 300 µl Protein A-Sepharose (50% suspension, Invitrogen) for 1 h at room temperature. Pelleted beads ($3000 \times g$, 5 min) were washed twice with sodium borate (0.1 mol/l, pH 9.0) and dimethylpimelidate (Sigma) was added at a final concentration of 20 mmol/l. After 30 min of incubation at room temperature, beads were spun down, washed once in ethanolamine (0.2 mol/l, pH 8.0), and subsequently incubated for 2 h in ethanolamine at room temperature. Beads were separated from unbound antibodies and resuspended in 120 µl phosphate-buffered saline containing 0.03% sodium azide.

Immunoprecipitation was performed as described before.[32] Briefly, 2 mg of protein extract was precleared with 10 µl of Protein A-Sepharose and subsequently incubated overnight at 4 °C with 40 µl of anti-GFP antibodies covalently linked to Protein A-Sepharose beads. Beads were pelleted, washed intensively, and bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a gradient gel (4–15%), followed by immunoblotting with an affinity-purified polyclonal rabbit anti-PDGF-D antibody (a kind gift from U Eriksson, Stockholm, Sweden).

Glomeruli, isolated from transgenic or wild-type mice, were lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting was performed as described[5] using a mouse monoclonal anti-EGFP antibody (Clontech, clone JL8) and a horseradish peroxidase-conjugated anti-mouse secondary antibody (vector).

TUNEL staining

TUNEL staining was performed in methacarn-fixated tissues using the ApopTag Plus fluorescein *in situ* apoptosis detection kit (Chemicon) according to the manufacturer's instructions.

Statistical analysis

All values were expressed as means ± s.d. Two-tailed tests as appropriate were used to compare the groups; that is, Student's *t*-test, Mann–Whitney *U*-test, or one-way analysis of variance with Bonferroni *post hoc* analysis. Statistical significance was defined as $P < 0.05$.

DISCLOSURE

CEA is a consultant with ZymoGenetics. All the other authors declared no competing interests.

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